Defining the Role of Notch Signaling in Vascular Smooth Muscle Cell Differentiation

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Abstract

Complications resulting from vascular disease such as atherosclerosis are a major health concern in the United States. Treatments to remove atherosclerotic plaques from occluded blood vessels often result in injury causing aberrant vascular smooth muscle cell (VSMC) proliferation leading to restenosis and vessel occlusion, rendering the procedure ineffective. Methods to prevent restenosis are limited due to the lack of understanding of the signaling pathways involved. The Notch signaling pathway is known to be important in VSMC development and likely contributes to vascular diseases, such as atherosclerosis and arteriosclerosis, and may be involved in the restenosis process. This study will define the actions of Notch signaling, and its interaction with two prominent signaling pathways, Platelet Derived Growth Factor (PDGF), which promotes proliferation and dedifferentiation and Transforming Growth Factor Beta (TGFß), which induces differentiation of VSMC. To investigate Notch signaling in vascular cells, human aortic smooth muscle cells were cultured and treated with pathway activators and inhibitors. RNA and protein was isolated from cultured cells and Western blots and quantitative polymerase chain reactions were used to measure the effects on smooth muscle differentiation markers. Results from this study indicate that PDGF in conjunction with Notch signaling promotes dedifferentiation and TGFß in conjunction with Notch activity promotes differentiation in VSMC. Differentiation and dedifferentiation was reduced when Notch signaling was inhibited. The conclusions from this research study have deepened the understanding of Notch signaling in vascular smooth muscle cells, and can possibly lead to the development of novel drug therapies to prevent restenosis.

Introduction

Complications resulting from atherosclerosis are one of the leading causes of death in the United States.[7] These complications include cardiovascular disease, myocardial infarction and stroke. The primary function of mature vascular smooth muscle cells is to maintain vascular tone and regulate blood pressure.[2] Defects in vascular smooth muscle cells is a contributing factor in cardiovascular diseases as well as atherosclerosis. Normal quiescent and mature vascular smooth muscle cells are non-proliferative and fully differentiated; expressing genes required for their contractile function.[2] A vascular smooth muscle cell in a proliferative state has proven to be a clinical issue in mature blood vessels. For instance, balloon angioplasty is a procedure used to remove atherosclerotic plaque from an occluded blood vessel. In the process of removing the atherosclerotic plaque the vessel wall is injured, which can result in smooth muscle cell proliferation leading to restenosis and vessel occlusion, rendering the procedure ineffective. Restenosis is the reoccurrence of stenosis or narrowing in a blood vessel after it has been treated with apparent success. Methods to prevent restenosis are limited due to the lack of understanding of the signaling pathway involved.

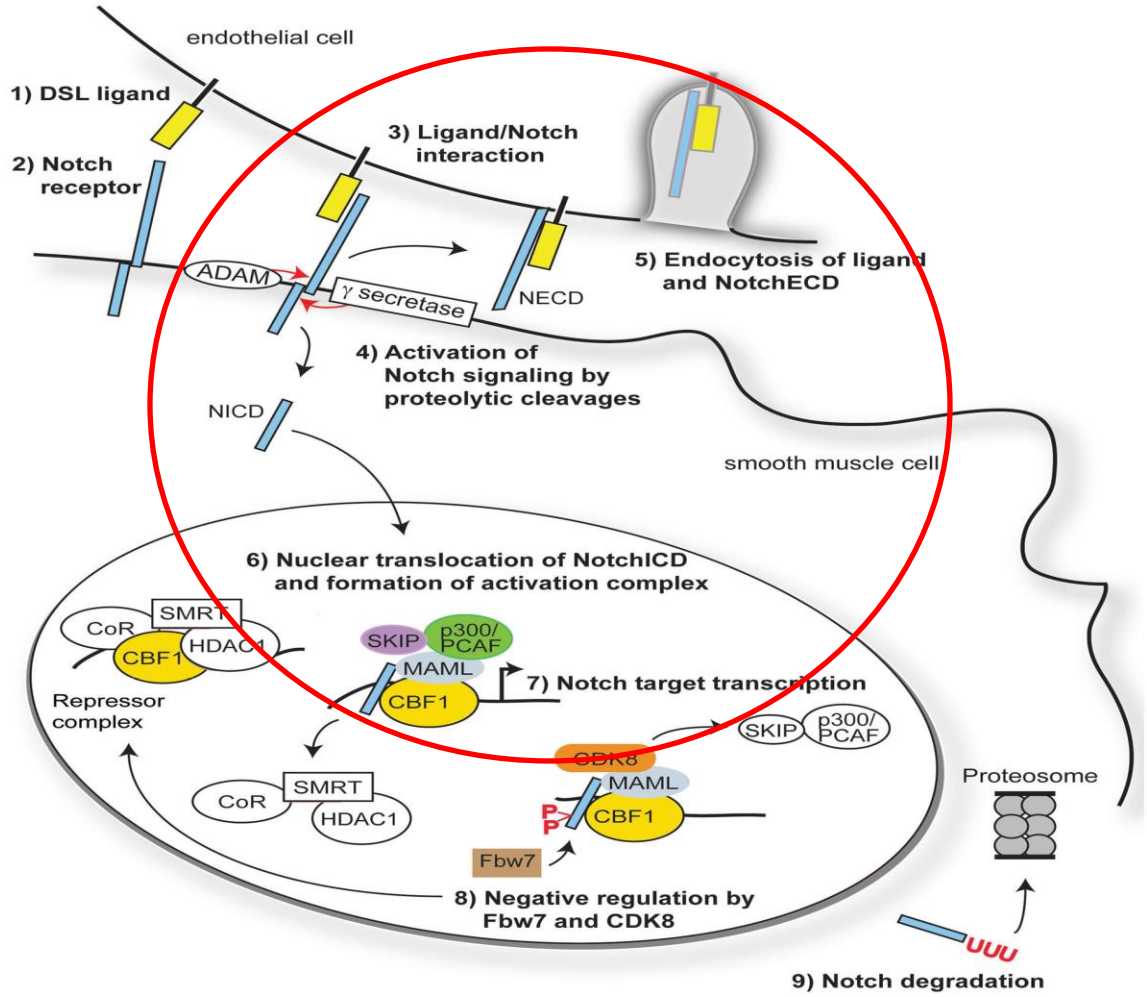
The evolutionarily conserved, Notch signaling pathway, is known to be important in vascular smooth muscle cell development, proliferation, migration and the development of vascular diseases such as atherosclerosis and arteriosclerosis.[5] Notch signaling receptors are also expressed throughout the lifespan of VSMC. Mice depleted in Notch1, Notch2, and Notch3 all present with vascular defects. [2] Despite its prominent role in vascular smooth muscle cell physiology, the pathway is not completely characterized. Furthermore, the role it plays in smooth muscle proliferation and restenosis is not clear. Published literature presents conflicting data on whether Notch signaling causes differentiation or dedifferentiation in vascular smooth muscle cells, suggesting complexity and potential interaction with other mediators. Currently, the Notch

signaling pathway is believed to be activated when a transmembrane ligand interacts with the extracellular domain of Notch receptors. This causes the receptor of an adjacent cell to change shape which exposes sites for cleavage of the receptors by the enzymes ADAM17 and γ-secretase. This cleavage results in liberation of the intercellular domain and translocation into the nucleus of the cell, where transcription of Notch genes are induced, (figure 1).[2] One way to inhibit Notch signaling activity experimentally is by the addition of DAPT. DAPT targets the γ-secretase enzyme preventing cleavage of the intracellular domain.

A study found strong expression of both Notch2 and Notch3 receptors in arteries derived from normal human lung biopsies while Notch1 was undectable.[2] As a result, this study attempts to uncover differences in the actions of Notch signaling, specifically Notch3, and its interaction with prominent signaling pathways in vascular smooth muscle cells. Two pathways were studied in conjunction with the Notch3 signaling pathway. Platelet Derived Growth Factor (PDGF), which promotes proliferation and dedifferentiation and Transforming Growth Factor Beta (TGFß), which induces differentiation of vascular smooth muscle cells.[3, 7] PDGF has been found to play a role in proliferative vascular smooth muscle diseases. PDGF is one of the primary agents that induce the synthetic phenotype of VSMC from the quiescent contractile phenotype, leading to the development of atherosclerosis.[3] TGFß plays a critical role in the pathogenesis of cardiovascular diseases, and regulates differentiation of VSMC. TGFß has been found to affect endothelial cells, monocytes, macrophages, platelets and other cells present in atherosclerotic and restenosis lesions.[7] Exploring the relationship between growth factors and Notch signaling may give insight into the causation of the restenosis process.

Notch, TGFß, and PDGF all influence VSMC, suggesting that they might interact to promote unique phenotypes in VSMC. Therefore, I proposed to ask how the Notch signaling

pathway affects the ability of PDGF to promote dedifferentiation, and TGFß to induce differentiation of vascular smooth muscles cells. I postulated that Notch signaling modulates the actions of PDGF and TGFß on vascular smooth muscle cells to alter their phenotype and function.



Joshua Boucher, Thomas Gridley, and Lucy Liaw. "Molecular pathways of Notch signaling in vascular smooth muscle cells." Frontiers in Physiology. 2012, Vol 3, Article 81

Figure 1. Notch Signaling Pathway. Notch signaling pathway is activated when the transmembrane ligand interacts with the extracellular domain of Notch receptors. The receptor undergoes a conformational change which exposes sites for cleavage of the receptors ADAM17 and γ-secretase. Cleavage results in liberation of the intercellular domain and translocation into the nucleus of the cell, where it induces transcription of Notch genes. DAPT experimental treatment can inhibit Notch signaling by targeting the γ-secretase enzyme and preventing cleavage of the intracellular domain.

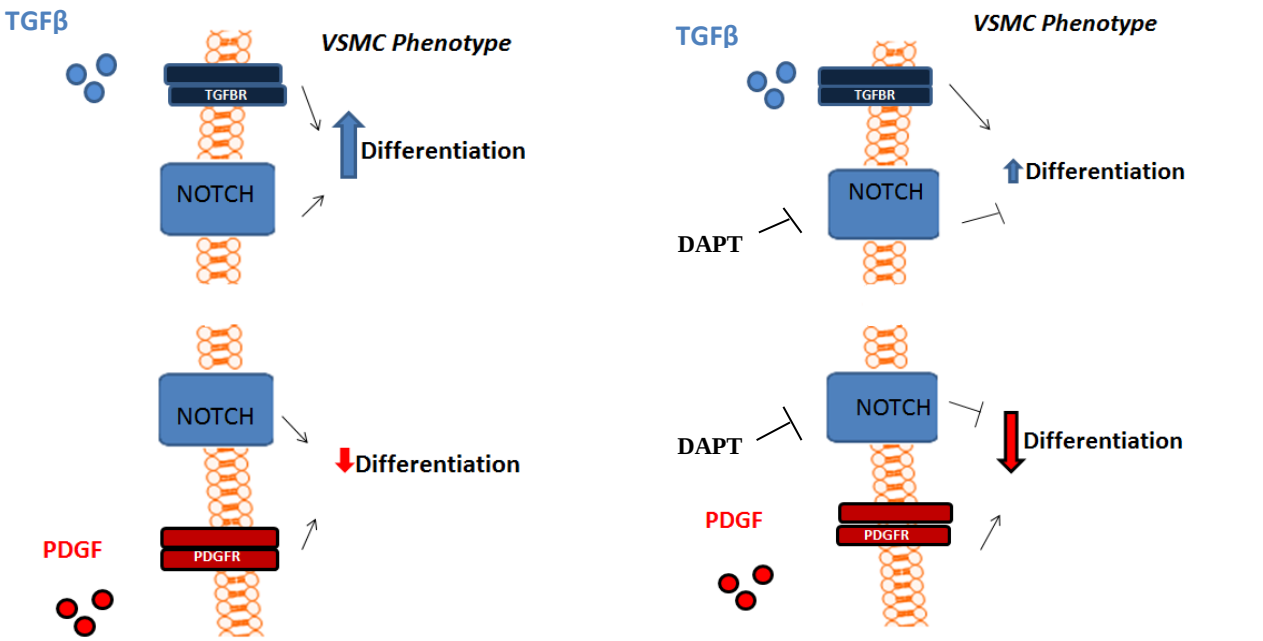


Figure 2. Research Hypothesis Notch signaling may modulate the ability of PDGF to promote dedifferentiation, and TGFß to induce differentiation of vascular smooth muscle cells. The inhibition of Notch3, by DAPT will weaken the growth factors' (PDGF and TGFß) ability to alter vascular smooth muscle cell phenotype and function.

Materials and Methods

Cells and Experimental Treatment

All of the experiments were conducted in human aortic smooth muscle cell cultures, and were grown in Dulbecco modified Eagle's minimal essential medium (DMEM media) and Fetal Bovine Serum (FBS), which is commonly used in cell culture. To test the interaction between PDGF and Notch signaling, human aortic smooth muscle cells were serum starved by culturing in 0.2% Fetal Bovine Serum and DMEM media for two days then were treated with 10ng/µl of PDGF growth factor and 10ng/µl of the Notch inhibitor, DAPT. DAPT targets the ɣ-secretase enzyme and inhibits the cleavage of the intracellular domain of the Notch Signaling Pathway, [2] (Figure 1). To uncover the interaction between TGFß and Notch, 10ng/µl of TGFß growth factors and DAPT was added to the human aortic smooth muscle cells. Treatments were added in 24 hour and 48 hour time courses. Cells that did not receive treatment served as the control.

Dimethyl Sulfoxide (DMSO) was the vehicle for DAPT. An equal amount of DMSO was added to cells that did not receive DAPT treatment. According to literature 10ng/µl is an acceptable dose for the growth factors and inhibitors.

RNA Extraction (Trizol Method)

Treated cells were trypsinized by TRYPLE Express Trypsin and incubated at 37 ^{o}C for 5 minutes. 5% FBS DMEM media was added to the cells to inhibit the action of trypsin. Cells were centrifuged and the supernatant was removed. The resulting pellet was suspended in Ribizol reagent and incubated at 25 ^{o}C for 5 minutes. Chloroform was added to the extract and incubated for 3 minutes. The extract was then centrifuged for 15 minutes at 4 ^{o}C. The upper aqueous phase was removed and placed in tubes containing 2 µl of glycogen. Isopropanol was added to the samples and incubated for 10 minutes and centrifuged for 10 minutes at 4 ^{o}C. The resulting RNA pellet was washed with 75% ethanol and centrifuged for 5 minutes at 4 ^{o}C. The RNA pellet was dried for 10 minutes at 37 ^{o}C and resuspended in nuclease free TE buffer. The RNA concentration was quantified using the NanoDrop Spectrophotometer.

Reverse Transcription Polymerase Chain Reaction (RT Reaction)

To 2 µl of RNA (25ng/µl) extracted by the Trizol method was added 18 µl of master mix (Invitrogen 5x Strand Buffer, 0.5µg/µl Random Primers, 10mM dNTP, 0.5 RNase Inhibitor, 0.5 µl M-MLV Reverse Transcriptase and molecular grade water). Samples were incubated at 37^{o}C for 2 hours. After incubation, 70µl of nuclease free water was added to the samples resulting in cDNA.

Quantitative Polymerase Chain Reactions

Relative gene expression was measured by primers for smooth muscle cell differentiation genes; calponin, Notch3, α-smooth muscle actin, smooth muscle myosin heavy chain, and SM22α. Primer sets contained both forward and reverse primers at a concentration of 50ng/µl. Master Mix (5 µl of SYBR Green and 0.5 µl of primer set) was added to 4.5 µl of diluted cDNA. Samples were run in duplicate, expression levels were normalized to GAPDH expression and compared by $2^{\Delta\Delta CT}$ results.

Protein Extraction

Treated cells were typsinsized by TRYPLE Express Trypsin and incubated at 37 ^{o}C for 5 minutes. 5% FBS DMEM media was added to the cells to inhibit the action of trypsin. Cells were centrifuged and the supernatant was removed. Cells were resuspended in RIPA Buffer (phenylmethanesulfonyl fluoride (PMSF) 1x100, Protease Inhibitor 1x100) and chilled for 20 minutes at 4 ^{o}C. The samples were centrifuged for 5 minutes at 4 ^{o}C and the supernatant containing protein was removed. Protein concentration was tested using the Bradford Assay. Samples were diluted with RIPA Buffer to contain a protein concentration of 20µg/µl. To the samples were added 15 µl of 5x loading dye and samples were boiled for 5 minutes and were stored at -20 ^{o}C.

Protein Immunoblot (Western Blot)

Protein extracted from cultured vascular smooth muscle cells was tested by Western blotting to identify protein expression as a result of the experimental treatment. 10% sodium dodecyl sulfate (SDS) polyacrylamide gels were prepared, samples were added and run in 10X

Run Buffer (Glycine, Tris Buffer, 10% SDS and distilled water). The gel was transferred to an Amershan Hybound ECL Nitrocellulose Membrane in Western Transfer Buffer (Glycine, Tris Buffer, 20% Methanol, and 0.5% SDS). The membrane was stained with Ponceau stain (1% Ponceau in 5% Acetic Acid). The membrane was blocked in milk (carnation 5% nonfat dry milk in TBS-T solution) for one hour, washed 3X 10 minutes in TBS-T solution, then blotted with primary antibodies, Notch3 rabbit antibody (1:1,000), α-smooth muscle actin rat antibody (1:2,000) and tubulin rat antibody (1:20,000) overnight. Membranes were washed 3X 10 minutes in TBS-T solution, and then blotted with rat and rabbit secondary antibody (1:5,000). The membranes were washed with TBS-T, added to a western blot cassette, where chemiluminescence reagents were added. Tubulin served as a protein loading control for the Western Blot assay.

Data Analysis

Results from the gPCR were normalized to GAPDH expression and compared in groups of four. Results were averaged and displayed with standard deviations. ANOVA was used to test for statistical significance using a p-value of 0.05.

Results

Platelet Derived Growth Factor (PDGF) induces reduction in vascular smooth muscle cell differentiation.

Results from qPCR of smooth muscle differentiation markers, calponin (CNN1) and α-smooth muscle actin (SMA) in cells treated with PDGF, revealed a significant decrease in differentiation compared to the control (Figure 3). Western blotting confirmed a qualitative

decrease in α-smooth muscle actin protein intensity in cells treated with PDGF compared to the control. Smooth muscle differentiation markers, smooth muscle myosin heavy chain (SMMHC) and sm22α (SM22), did not reveal a significant difference in expression compared to the control in the 24 hour time course (Figure 3A).

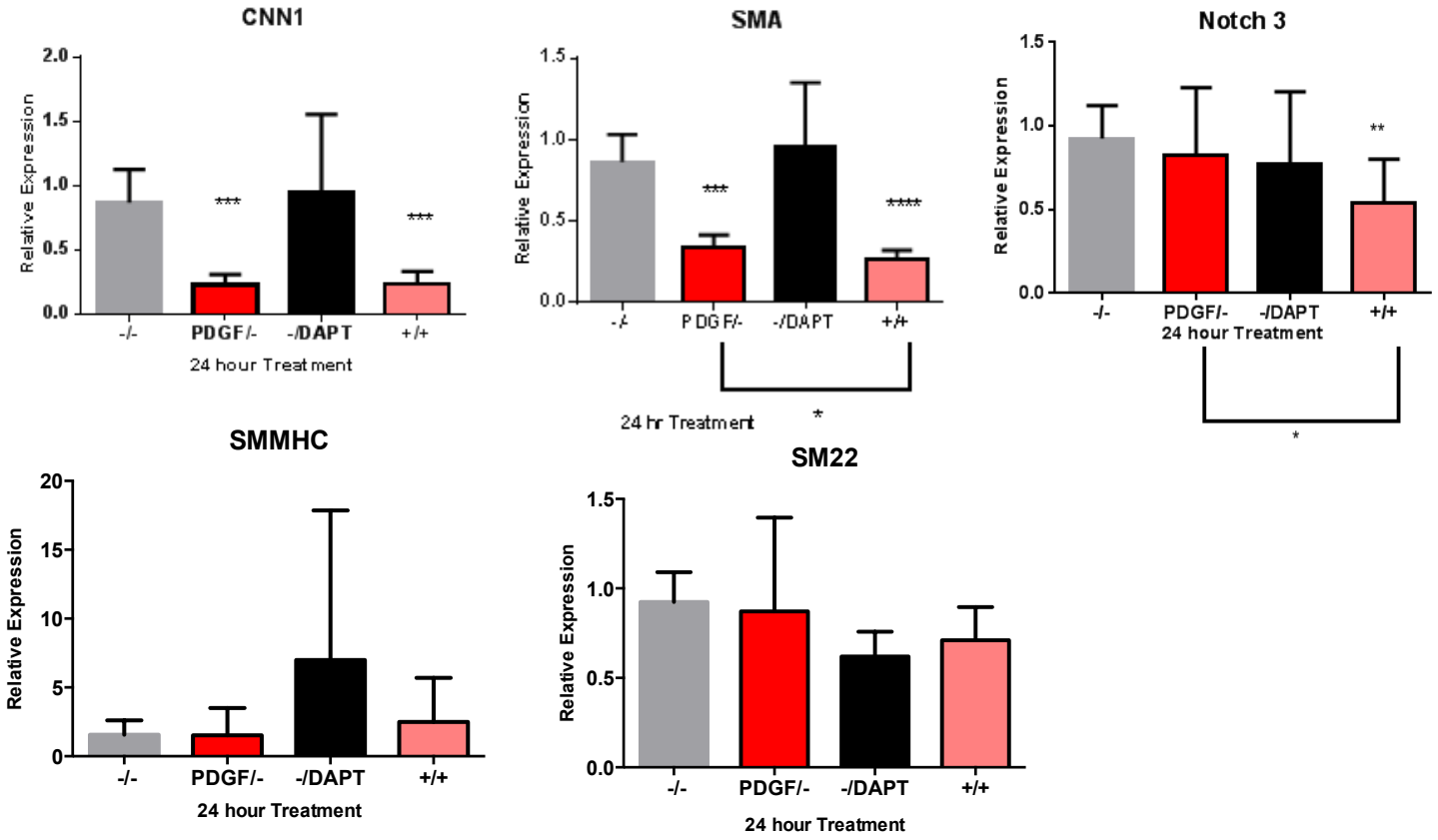
Low level of Notch Signaling in cultured vascular smooth muscle cells.

Cells treated with the Notch inhibitor DAPT alone did not produce a significant difference compared to the control in vascular smooth muscle cell differentiation genes, CNN1, SM22α, SMMHC, and SMA. Quantitative Polymerase Chain Reaction results for Notch3 gene expression revealed no significant decrease in cells treated with DAPT alone (Figure 3A) in the PDGF experiments. In the TGFß experiments, there was a significant difference in Notch gene expression in cells treated with DAPT (Figure 4A). Western blotting revealed a qualitative decrease in Notch3 protein intensity in cells treated with DAPT compared to control in both the PDGF and TGFß experiments, (Figure 3B, 4B).

Inhibition of Notch by DAPT and addition of PDGF resulted in greater reduction in vascular smooth muscle cell differentiation.

Cells treated with DAPT and PDGF showed a significant decrease in differentiation in α-smooth muscle actin (SMA) gene expression as well as in Notch3, (Figure 3A). In SMA gene expression, there was a significant decrease in differentiation in cells treated with PDGF and DAPT compared to the cells treated with PDGF alone. In Notch3 gene expression, the only significant difference was seen in cells treated with both PDGF and DAPT.

3A.



3B.

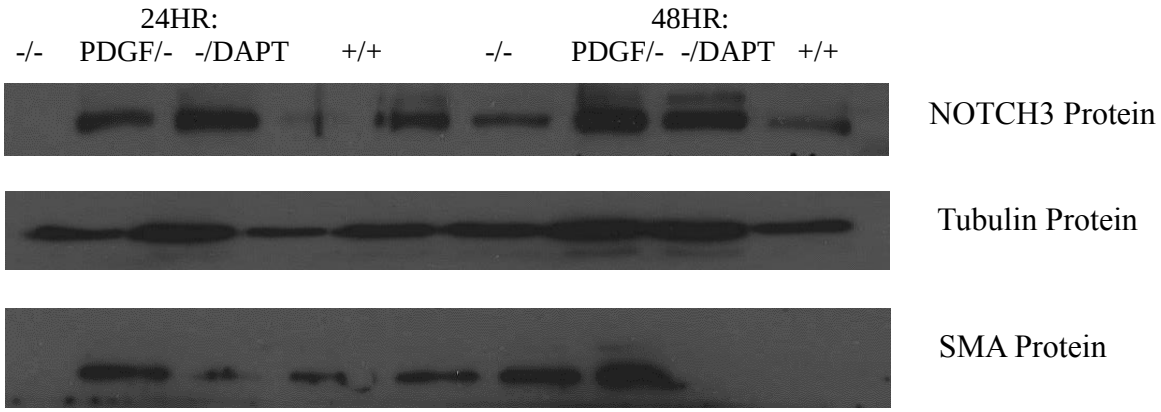


Figure 3A. Relative expression of vascular smooth muscle cell differentiation markers: Calponin (CNN1), NOTCH3, α-Smooth Muscle Actin (SMA), Smooth muscle myosin heavy chain (SMMHC) and Sm22α (SM22). Control denoted as (-/-). Combined PDGF and DAPT treatment denoted as (+/+). Significance denoted by asterisks (*p-value<0.05, **p-value <0.01, ***p-value <0.001, **** p-value<0.00001) and standard deviation denoted by figure bars. (n=5). 48 hour time course found in supplemental data. **B.** Western blot data for Notch3 and SMA. Tubulin served as a loading control.

1.

Gene	treatment	$2^{\Delta\Delta CT}$ Mean Difference	Standard Deviation	P-Value
CNN1	PDGF/-	0.2261	0.08103	0.0004 *
CNN1	-/DAPT	0.9489	0.6069	0.9525
CNN1	+/+	0.2338	0.09684	0.0008 *
SMA	PDGF/-	0.3373	0.07728	0.0001 *
SMA	-/DAPT	0.9564	0.3934	0.8786
SMA	+/+	0.2643	0.05684	<0.0001 *
Notch3	PDGF/-	0.8257	0.4039	0.8340
Notch3	-/DAPT	0.7705	0.4341	0.6815
Notch3	+/+	0.5369	0.2634	0.0064 *
SMMHC	PDGF/-	1.525	1.981	>0.9999
SMMHC	-/DAPT	6.991	10.86	0.4387
SMMHC	+/+	2.489	3.209	0.8255
SM22	PDGF/-	0.8720	0.5231	0.2981
SM22	-/DAPT	0.6194	0.1392	0.6714
SM22	+/+	0.7110	0.1845	0.5856

Table 1. PDGF 24 hour time course statistics. Significant difference (p-value<0.05) denoted with an asterisk

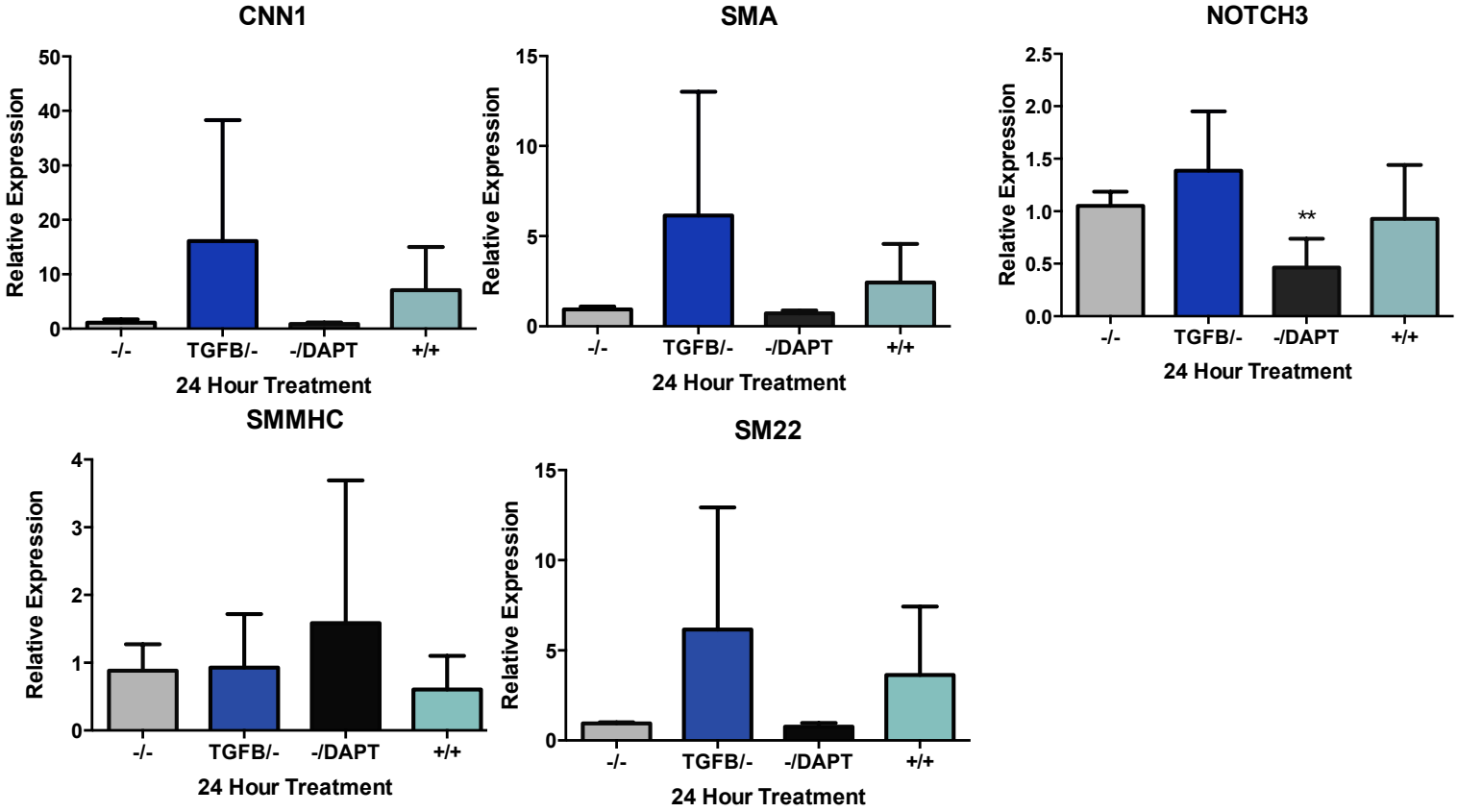
Transforming Growth Factor (TGFß) increases differentiation in vascular smooth muscle cells.

Results from qPCR of smooth muscle differentiation markers, calponin (CNN1), α-smooth muscle actin (SMA), and sm22α (SM22) of cells treated with TGFß, revealed an increase in differentiation compared to the control, although one-way Anova did not show a significant difference due to a large standard deviation (Figure 4A). Western blotting confirmed a qualitative increase in α-smooth muscle actin protein intensity in cells treated with TGFß compared to the control (Figure 4B).

Inhibition of Notch by DAPT and addition of TGFß resulted in a smaller increase in vascular smooth muscle cell differentiation.

Cells treated with DAPT and TGFß showed a smaller increase in differentiation in all of the smooth muscle cell differentiation markers, although the one way Anova did not reveal a significant difference due to a large standard deviation. There was no significant difference in Notch3 gene expression (Figure 4A, 4B).

4A.



4B.

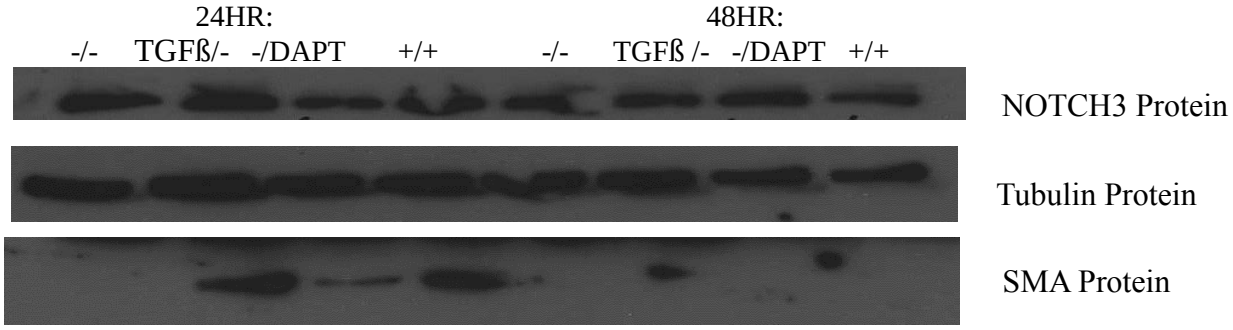


Figure 4A. Relative expression of vascular smooth muscle cell differentiation markers: Calponin (CNN1), NOTCH3, α-Smooth Muscle Actin (SMA), Smooth muscle myosin heavy chain (SMMHC) and Sm22α (SM22). Control denoted as (-/-). Combined TGFß and DAPT treatment denoted as (+/+). Significance denoted by asterisks (**p-value <0.01) and standard deviation denoted by figure bars. (n=3). 48 hour time course found in supplemental data. **B.** Western blot data for Notch3 and SMA. Tubulin served as a loading control.

2.

Gene	treatment	$2^{\Delta\Delta CT}$ Mean Difference	Standard Deviation	P-Value
CNN1	TGFβ/-	2.403	1.658	>0.05
CNN1	-/DAPT	0.9368	0.7011	>0.05
CNN1	+/+	1.551	1.058	>0.05
SMA	TGFβ/-	0.5974	0.5974	0.9946
SMA	-/DAPT	0.8974	0.09572	0.9887
SMA	+/+	0.8641	0.04786	0.9875
Notch3	TGFβ/-	1.487	0.5111	0.2407
Notch3	-/DAPT	0.5213	0.2999	0.0349
Notch3	+/+	0.8953	0.5103	0.8727
SMMHC	TGFβ/-	0.9275	0.7903	0.9975
SMMHC	-/DAPT	1.584	2.107	0.7345
SMMHC	+/+	0.6034	0.4994	0.5035
SM22	TGFβ/-	6.155	6.77	0.2527
SM22	-/DAPT	0.7689	0.1999	0.1795
SM22	+/+	3.630	3.797	0.3024

Table 2. TGFß 24 hour time course statistics. Significant difference (p-value<0.05) denoted with an asterisk

Discussion

Results of the study confirmed that Platelet Derived Growth Factor (PDGF) induces dedifferentiation in vascular smooth muscle cells (VSMC). As hypothesized, PDGF reduced differentiation in treated cells measured by smooth muscle differentiation genes calponin and α-smooth muscle actin. Unexpectedly, smooth muscle differentiation genes, sm22α and smooth muscle myosin heavy chain did not reveal a significant decrease in differentiation. PDGF may affect these genes differently than calponin and α-smooth muscle actin.

The inhibition of Notch signaling by DAPT alone did not produce a substantial difference in smooth muscle differentiation. DAPT is known to be a strong inhibitor of Notch signaling in other cell cultures such as endothelial cell cultures. The data suggests that Notch activity may be low in cultured human aortic VSMC. Even with low Notch activity present in the cultured VSMC, a significant decrease in smooth muscle actin gene expression was seen in cells treated with both PDGF and DAPT, compared to PDGF treated cells, and a greater decrease in gene expression compared to the control. This result indicates that Notch signaling may interact with PDGF. To further reinforce this result, cells treated with both PDGF and DAPT showed a significant decrease in Notch3 gene expression compared to the control. Western blotting also showed a decrease in Notch3 and α-smooth muscle actin protein intensity in DAPT treated cells, as well as in cells treated with both PDGF and DAPT. Notch3 and α-smooth muscle actin were the only proteins analyzed using western blotting due to the availability of primary antibodies.

Results of the study also confirmed that Transforming Growth Factor (TGFß) induces differentiation in vascular smooth muscle cells (VSMC). As hypothesized, TGFß increased differentiation in treated cells measured by smooth muscle differentiation genes calponin, α-smooth muscle actin and sm22α. Unlike the other differentiation markers, smooth muscle

myosin heavy chain gene expression was not greatly increased; indicating TGFß may interact with this gene differently than the others. Due to a large standard deviation, no significant difference was found in the TGFß treated cells.

As in the PDGF experiments, there proved to be low Notch activity in the human aortic cultured cells, although there was a significant decrease in Notch3 gene expression in the cells treated with DAPT in the TGFß experiments. This decrease was not maintained during the 48 hour time course. Combined TGFß and DAPT treatment resulted in a smaller increase in differentiation as hypothesized, although a large standard deviation prevented a significant difference to be found. Results of the TGFß treatment did not reinforce an interaction between TGFß and Notch signaling.

Further studies must be conducted to uncover the relationship between PDGF, TGFß and Notch signaling. A future study may overexpress Notch signaling in VSMC to potentiate the relationship between the growth factors and Notch signaling. In all, results of this study showed that PDGF induces dedifferentiation in VSMC and TGFß induces differentiation of VSMC. The results also show that these growth factors act in expected ways to regulate smooth muscle differentiation genes. Inhibition of Notch signaling by DAPT in the presence or absence of these growth factors did not have a substantial effect on their differentiation and dedifferentiation capabilities. Although in the combined presence of PDGF and DAPT there was a greater decrease in Notch3 and α-smooth muscle actin expression. The data suggest that Notch signaling may interact with PDGF signaling to control smooth muscle differentiation. The relationship between PDGF and Notch signaling may lead to drug therapies that prevent atherosclerosis and the restenosis process.

Acknowledgments

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References

1. Matthew R. Alexander and Gary K. Owens. "Epigenetic Control of Smooth Muscle Cell Differentiation and Phenotypic Switching in Vascular Development and Disease." The Annual review of Physiology. 2012. 74:8.1–8.28.

2. Joshua Boucher, Thomas Gridley, and Lucy Liaw. "Molecular pathways of Notch signaling in vascular smooth muscle cells." Frontiers in Physiology. 2012, Vol 3, Article 81.

3. Johanna Donovan, David Abraham, Jill Norman. "Platelet-derived growth factor signaling in mesenchymal cells." Frontiers in Bioscience. 2013; Landmark, 18, 106-119.

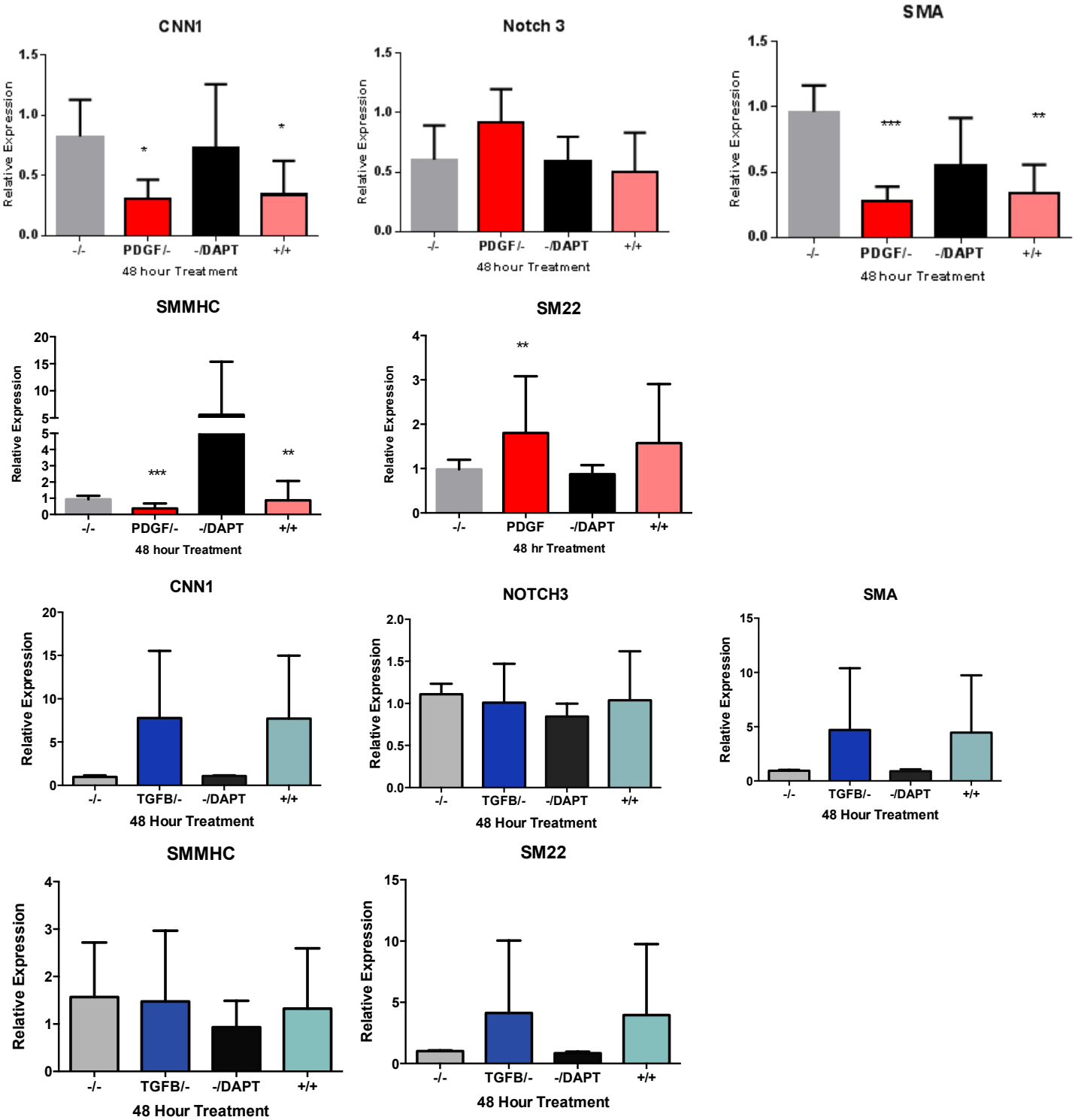
4. Konstantin Gaengel, Guillem Genové, Annika Armulik and Christer Betsholtz, "Endothelial-Mural Cell Signaling in Vascular Development and Angiogenesis." Arteriosclerosis, Thrombosis, and Vascular Biology: American Heart Association. 2009.

5. Thomas Gridley. "Notch signaling in vascular development and physiology." Development . 2007. 134, 2709-2718.

6. Elaine W. Raines. "PDGF and cardiovascular disease." Elsevier. Cytokine & Growth Factor Reviews. 15. 2013, 237-254.

7. Evangelia Pardali, Peter ten Bijke. "TGFB Signaling and Cardiovascular Diseases." International Journal of Biological Sciences. 2012; 8(2):195-213.

Supplemental Data:



Supplemental Data: Relative expression of vascular smooth muscle cell differentiation markers: Calponin (CNN1), NOTCH3, α-Smooth Muscle Actin (SMA), Smooth muscle myosin heavy chain (SMMHC) and Sm22α (SM22). Control denoted as (-/-). Combined treatment denoted as (+/+). Significance denoted by asterisks (*p-value<0.05, **p-value <0.01, ***p-value <0.001) and standard deviation denoted by figure bars.